value of 5·8, within the sampling error, for the total pH of the yeast cell. This in turn provides evidence for the view that the adjusted pK values of 4·6 and 6·2 for acetic and carbonic acids are at least approximately correct for the *in vivo* conditions of the cell. After freezing in liquid air or oxygen and without monoiodoacetate, the thawing to room temperature should be done rapidly and the pH then measured without further delay, as it falls slowly with time.

The average value of 5·8 for the intracellular pH is somewhat subject to the previous history of the yeast. Thus we have found that after prolonged oxygenation (8–48 hr.) it is approximately 6.

The pH of the small outer region, or cell wall (Conway & Downey, 1950), seems in the resting state to be about the same as for the whole cell.

During fermentation marked pH changes occur in the yeast cell. There is a considerable rise of the total or overall pH (carbon dioxide being evacuated). This increase of the pH was shown with 0·1 M-potassium chloride in the suspending fluid, but it also occurs without potassium chloride in the suspending fluid but to a somewhat lesser degree (shown in a further communication). Increase in intracellular pH during fermentation was also observed by Brandt (1945).

At the same time there is a marked fall in the pH value of the outer region or cell wall. Such changes have an important bearing on the mechanism of the production of acidity by yeast, which in turn has a general biological application.

When yeast is suspended in acetic acid solution (1 : 1) the acid enters very rapidly, and when in 0·2 M concentration would appear to have fully entered in less than 1 min. Under the given conditions there is but little metabolic disappearance of the acetic acid, and when suspended in water some volatile acid, other than carbon dioxide and possibly acetic, appears outside the cells.

SUMMARY

1. The overall intracellular pH of resting baker's yeast was determined by three methods, a glass electrode method after freezing with liquid air and thawing out, a carbon dioxide method and an acetic acid method. The mean values obtained were 5·80 ± 0·02 (eighteen determinations), 5·81 ± 0·05 (six determinations) and 5·81 ± 0·02 (twenty-six determinations).

2. The resting pH of the outer region (cell wall) was determined by the distribution of glyceric acid at various concentrations after suspending in glyceric acid solutions. The resting value appears to be the same or nearly the same as for the whole cell.

3. When yeast ferments glucose this outer region becomes markedly acidic and the inner part of the cell more alkaline than in its resting state.

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Biological Production of Acid and Alkali

1. QUANTITATIVE RELATIONS OF SUCCINIC AND CARBONIC ACIDS TO THE POTASSIUM AND HYDROGEN ION EXCHANGE IN FERMENTING YEAST

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When Pulver & Verzár (1940) showed that during short period fermentation potassium was absorbed by yeast and released again towards the end of fermentation, the occurrence appeared to be readily interpretable in terms of the process of potassium accumulation in muscle and other animal cells (Conway & Boyle, 1939; Boyle & Conway, 1941; Conway, 1945; Conway, FitzGerald & MacDougald, 1946; Conway, 1947*a*). It was quickly found that this special form of potassium absorption in yeast was associated with a high degree of acid formation (Conway & O'Malley, 1942). Direct exchange of K^+ and H^+ ions was recognized, but not considered as the main process until somewhat later (Conway & O'Malley, 1943), when it was shown that a pH as low as 1·7 could be produced by adjusting the con-

ditions. It is now known that a pH of 1·5–1·6 is readily obtainable, and 1·4 has been observed on a few occasions. It was also shown that a similar exchange of H^+ for NH_4^+ ions occurred in the special 'ammonia' yeast, in which all the potassium in yeast was replaced by ammonium ions (Conway & Breen, 1945). Various quantitative data were published by Conway & O'Malley (1944), and a full account of experimental conditions some time later (Conway & O'Malley, 1946).

Rothstein & Enns (1946) described experiments from which the same general conclusion could be drawn, that the K^+ absorption occurred as a direct exchange for H^+ ions, and it would appear had arrived at such a view as early as 1943 from their experimental data.

Pursuing the question here as to the origin of the H^+ ions exchanging for K^+ ions, it became clear that the acidity produced by the fermenting yeast in an unbuffered solution was to be regarded under two headings, (*a*) organic acid excretion or its appearance outside the cells; (*b*) the K^+ and H^+ exchange when potassium chloride is added with formation of considerable free hydrochloric acid.

In unbuffered solution with 1 kg. of washed centrifuged yeast suspended in 0·6 l. of 5% (w/v) glucose, about 40–50 m-equiv. organic acid per litre of external fluid appeared in the fluid outside the cells after 0·5 hr. at room temperature. This organic acid proved to be almost entirely succinic acid. If potassium chloride to the extent of about 100 mmol./l. was incorporated in the glucose solution, the succinic acid was much reduced in amount and free hydrogen ions to the extent of about 20 m-equiv. appeared, the total titratable acidity being increased, but usually only to a small extent.

Here it seemed that succinic acid was a major source of the H^+ ions exchanged for K^+, potassium succinate being retained in the cells.

Further, it was found that if the yeast was oxygenated for a long time prior to the fermentation, there was a considerable fall in the succinic acid extruded, and in one experiment it was almost entirely absent. Yet under these conditions the free acid was formed with potassium chloride present, and by exchange of K^+ for H^+ ions reached maximum values. Clearly there was some other source of acidity, and it appeared that under these conditions extra bicarbonate was formed in the cells almost equivalent to the absorbed K^+ ions. Under these circumstances, also, the total amount of ether-extractable organic acids (after acidification) in the yeast was decreased and practically no increase occurred during fermentation, although the highest levels of free H^+ ion excretion were reached.

This indicated that succinic acid and carbonic acids could both contribute H^+ ions for the K^+ and H^+ ion exchange. After a long search they were the only sources of acidity, of quantitative significance, that we could find to account for the anions associated with the K^+ ions absorbed from an unbuffered solution. Furthermore, they were quantitatively adequate.

The next phase of the research into the mechanism of the exchange, which is dealt with in another paper, led, however, to the conclusion that though carbonic acid, in an indirect way, as will be discussed, could be held to supply H^+ ions in exchange for the absorbed K^+ ions, it was not the immediate or direct source. The source was still an organic acid, but such acid appears now to be the reduced form of a heavy metal catalyst, of which only a trace need be present at any time, operating in a cycle of reduction and oxidation across the cell membrane.

The present paper is mainly concerned with the quantitative relations of succinic and carbonic acids to the free acidity formed when K^+ exchanges for H^+ in fermenting yeast. Mention is also made of the search for other acids. Thus in a previous communication (Conway & O'Malley, 1946) it was pointed out that pyruvic acid formation went parallel with the change of pH, but it was found that only a very small fraction of the absorbed K^+ could be associated with pyruvate ions, using unbuffered media. Besides pyruvic and phosphopyruvic acids, many others have been determined in the fermenting suspensions, including succinic, citric, *iso*citric, aconitic, acetic and oxaloacetic acids. An investigation of the total ether-extractable acids has also been carried out.

METHODS

Experimental

The experiments throughout were carried out with baker's yeast as supplied by the Cork Yeast Factory. Fresh yeast was washed twice with ten times its volume of tap water, and the moist yeast, after the second centrifuging, used in the fermentation studies.

Oxygenated yeast. Such yeast was prepared by suspending 1 part in 6 parts of tap water, and O_2 bubbled through the mixture from a cylinder for several days.

Analytical

Hydrogen-ion concentration and total acidity of the suspending fluid. The hydrogen-ion concentration was determined both by glass electrode measurements of the pH and by electrometric titration and study of the titration curves, which also gave a measure of the total acidity and hence of the organic acid and its buffering power.

Total ether-extractable acids (in suspension or suspending fluid). 10 ml. portions of yeast suspension or suspending fluid were added to 5 ml. of 10N-H_2SO_4 in a Kutcher-Scheudel type of extractor (Krebs, Smyth & Evans, 1940). The high concentration of acid was used to ensure that the yeast cells would be rapidly ruptured. The extraction was continued for 9 hr. after which the ether extract was dried over anhydrous Na_2SO_4 and filtered. This treatment with Na_2SO_4 removes any mineral acid, which may have been

carried over during the extraction. After very thorough washing of the sodium sulphate with successive portions of dry ether, the combined ether extracts were evaporated to dryness, the residue dissolved in 10 ml. of hot water, cooled, and electrometrically titrated. After the titration the neutral solution was usually transferred to a 25 ml. volumetric flask, made up to volume and the succinic acid content determined using succinic dehydrogenase.

Keto acids. The method used was a modification of the colorimetric method developed by Lu (1939). The yeast cells were ruptured and two methods were employed for this purpose: (*a*) 2 ml. of yeast suspension were added to 10 ml. of boiling phosphate buffer, pH = 7·4, and the boiling continued for 3 min. The contents were then transferred quantitatively to a 50 ml. graduated cylinder containing 35 ml. of 0·1% dinitrophenylhydrazine in 2N-HCl, made up to 50 ml. and thoroughly shaken. 5 ml. of this suspension were used for the subsequent extraction. (*b*) The yeast suspension was immersed in liquid air. On thawing it was diluted as above with 35 ml. of 0·1% dinitrophenylhydrazine hydrochloride and made up to 50 ml.

Free pyruvic acid. In determining the free pyruvic acid by Lu's (1939) method we found it advisable to add a high proportion of dinitrophenylhydrazine, as indicated above, in order to saturate the other ketonic components.

Phosphopyruvic, pyruvic and total keto-acids. In order to determine the phosphopyruvic and other possibly phosphorylated ketonic acids it was necessary to remove the phosphorus by acid hydrolysis. For this purpose 1 ml. of yeast suspension was added to 40 ml. of 0·1% dinitrophenylhydrazine in 2N-HCl, heated to 80° and maintained at this temperature for 1 hr. On cooling, the contents were made up to 50 ml., and 5 ml. of the suspension was used for the determination.

Succinic acid. In the initial experiments, the chemical method as developed by Goepfert (1940) was used. Subsequently, the biological method using succinic dehydrogenase was employed (Weil-Malherbe, 1937; Krebs *et al.* 1940). Also we have found electrometric titration a very rapid and sufficiently accurate method for succinic acid determinations when, as in the suspending fluids after fermentation, other acids with similar ionization constants were present only in negligible amounts. On the electrometric titration curve the equivalents of alkali required to titrate from a pH of 4·0–5·5 is multiplied by 1·75 to convert to equivalents of succinic acid.

Citric, iso*citric and* cis-*aconitic acids.* The method used was that of Krebs & Eggleston (1944).

Acetic acid. This was determined by a steam-distillation method, the distillate being collected in successive fractions and titrated.

The carbon dioxide system. The CO_2 content of yeast fermenting under various conditions was determined by a microdiffusion procedure (Conway, 1947*b*; using no. 2 microdiffusion units). The fermentation conditions were the following (the volumes of suspending fluid, etc., being previously described):

(1) Yeast fermenting with exposure to the atmosphere.

(2) Yeast fermenting *in vacuo*. In these experiments the mixture was fermented in Thunberg tubes continuously exhausted by a water pump. In some cases cotton wool, impregnated with 10% (w/v) NaOH, was placed in the side arm to reduce further the CO_2 tension.

(3) Yeast fermenting in a stream of pure CO_2.

(4) Yeast fermenting in a stream of pure O_2.

For the *in vacuo* experiments 25 ml. of hot NaOH solution were run into each Thunberg tube containing 5 ml. of fermenting mixture at the end of the fermentation period, and the CO_2 then determined with suitable blanks. In some experiments samples were taken directly without introduction of alkali. With the experiments in which CO_2 was bubbled through the mixtures, the procedure has been described in a previous communication (Conway & Downey, 1950).

In these experiments, controls containing similar yeast suspensions without glucose, water being added in similar volume to the glucose solution, were set up, and also the gas was bubbled through water and KCl solution, the latter containing KCl in the same concentration as in the suspending fluid for those yeast suspensions which contained KCl.

Fermentation rate. The suspension consisted of 10 parts of moist yeast (by weight), 5 parts of tap water or 0·2M-KCl and 1 part of 30% (w/v) glucose.

After addition of the glucose solution, 0·5 ml. samples were added in a thin uniform layer to the outer chamber of no. 2 units. Immediately prior to sealing the unit 0·2 ml. of 0·17N-NaOH containing phenolphthalein as indicator was added to the central chamber, the lid quickly placed on the unit and this rotated once or twice. The time of sealing was noted, and the unit left absorbing for exactly 10 min. After removal of the lid two drops of saturated $BaCl_2$ solution were added to the NaOH solution to precipitate the carbonate and the unit immediately titrated with 0·2N-HCl.

RESULTS

The significance of succinic acid for the hydrogen-ion exchanges

The formation of succinic acid. It was found at an early stage, under the conditions of fermentation in which a high proportion of yeast to suspending fluid (1 : 0·6 or 1 : 1) existed, that much free organic acid was formed and diffused out of the yeast cells. With potassium chloride introduced into the suspending fluid and a corresponding marked fall in the pH during fermentation, there was a considerable diminution in the organic acid appearing in the outer medium. The curves of Fig. 1, summarizing three sets of experiments, illustrate the results of the electrometric titration of the suspending fluid with and without potassium chloride, expressed as m-equiv. of titrating alkali per litre of fluid.

In these experiments the total acid production is not much different when one includes potassium chloride, but it has changed in type, a considerable proportion appearing as free dissociated acid or as free hydrogen ions, with a corresponding fall in the organic acid.

The nature of the organic acid was then investigated, and it was found to be almost entirely succinic acid. The pure acid and sodium salt were isolated in practically quantitative amounts as determined by Goepfert's (1940) method and later by the succinic dehydrogenase method. Determinations by the Goepfert method showed that at least 90% of the organic acid appearing in the suspending fluid was

succinic acid, and the results with the succinic dehydrogenase method indicated that practically all the organic acid was present in this form. Also, the form of the electrometric titration curve of the suspending fluid agrees almost exactly with similar titration curves of pure succinic acid.

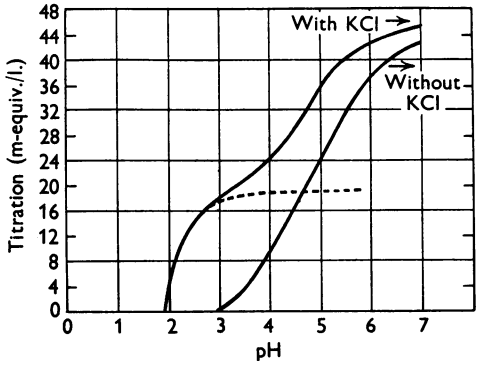

Fig. 1. Titration curves of supernatant fluid after fermenting 1 part of washed centrifuged yeast with 0·6 vol. of 5% (w/v) glucose containing 0·1 M-KCl, both for 40 min. at room temperature. Each curve is from the averaged data of three sets of experiments. The dotted line indicates the level of free HCl present.

Here attention may be drawn to the large amounts of succinic acid in the suspending fluid under the fermentation conditions, about 0·2% (w/v) free acid appearing in the unbuffered fluid external to the yeast cells after 30 min.

Succinic acid and the H^+ *and* K^+ *exchanges.* Experiments were then carried out in which the succinic acid formation, in the presence and absence of potassium chloride, was determined in the yeast cells (calculated from that in the suspending fluid and in the whole suspension) as well as in the centrifuged fluid. Such experiments are illustrated in Table 1.

In the presence of potassium chloride the succinic acid in the suspension as a whole differed little from that of the suspension without potassium chloride, the concentration being 14·4 m-equiv./l. as compared with 13·2 m-equiv./l. In the distribution of the succinic acid, there was, however, a marked difference. With potassium chloride the succinic acid in the suspending fluid was 7·6 m-equiv./l. less, and in the yeast cells 5·4 m-equiv./l. more than the corresponding figures without potassium chloride.

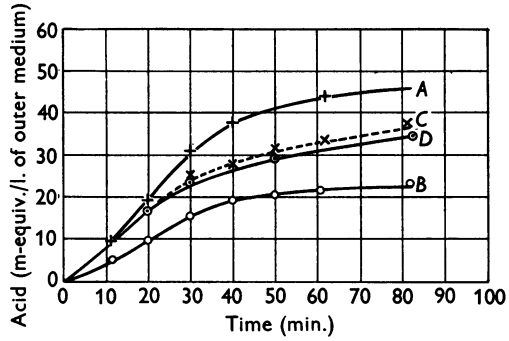

Fig. 2. Average curves of total acid and of succinic acid per l. of supernatant fluid after fermenting for different time periods. 1 l. of 5% (w/v) glucose, with and without the addition of 0·04 l. 2 N-KCl, with 1 kg. of centrifuged yeast. Room temperature. Three sets of experiments for each curve. Curve *A*, total acid with KCl; curve *B*, succinic acid with KCl; curve *C*, total acid without KCl; curve *D*, succinic acid without KCl.

Succinic acid was thus retained in quantity in the cells in the presence of potassium chloride. Assuming that the higher succinic acid content of the yeast cells supplies the anions associated with the K^+ ions exchanged for the H^+ ions and that only the first ionizing group of succinic acid is involved (pK = 4·2) then, under such conditions, about 50% of such associated anions can be accounted for by succinic acid.

The degree to which succinic acid is involved can vary much with the previous history of the yeast. As will be seen, after the yeast has been oxygenated for some days succinic acid contributes only a little to the acid formation.

Fig. 2 shows the course of the appearance of succinic acid and of the total titratable acid in the outer

Table 1. *Succinic acid content of yeast cells and of the outer medium after* 40 *min. fermentation*

(1 vol. of centrifuged yeast containing 0·2 vol. intercellular fluid to 0·5 vol. of water or 0·2 M-KCl, plus 0·1 vol. of 30% (w/v) glucose. The succinic acid concentrations are expressed throughout as m-equiv. with reference to 1 l. of the suspending fluid, or outer medium, which is also approximately the same as expressing them with reference to 1 kg. yeast cells, and about twice the value if referred to 1 l. of suspension.)

Without KCl			With KCl		
	Succinic acid			Succinic acid	
pH outer medium	Outer medium	Cells	pH	Outer medium	Cells
2·95	29·1	3·7	1·95	21·0	6·3
3·00	19·3	2·0	1·80	11·7	7·3
3·15	30·4	0·6	2·15	23·7	9·0
Mean **3·05**	**26·3**	**2·0**	**1·97**	**18·7**	**7·4**

medium from the beginning of fermentation (1 kg. of centrifuged yeast to 1 l. of 5% (w/v) glucose, with and without the inclusion of 40 ml. of 2N-potassium chloride).

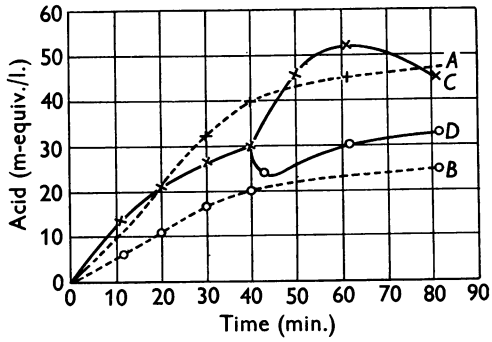

Fig. 3. Average curves of total acidity and of succinic acid as in Fig. 2, but with KCl added after 40 min. (0·04 l. of N-KCl) as well as at the beginning. Three sets of experiments for each curve. Curve *A*, total acid with KCl included from zero time; curve *B*, succinic acid with KCl included from zero time; curve *C*, total acid with KCl added after 40 min.; curve *D*, succinic acid with KCl added after 40 min.

It will be seen that without potassium chloride total titratable acid concentration is nearly identical with that of succinic acid in the suspending fluids. In these experiments the presence of potassium chloride results in the total acid being much increased and the succinic acid reduced by a nearly corresponding amount.

a fall in the external succinic acid concentration and a rapid rise in the free and total acidity. The rise in the free acid is much greater than the fall of the succinic acid. The dotted curves give the mean figures for total acid and succinic acid when the potassium chloride is included from the beginning of fermentation.

Figs. 4*a*, *b* show the course of the total acid and free succinic acid formation with 10 and 5% (w/v) glucose, and potassium chloride included from the beginning, the proportions being as for Figs. 1 and 2. It will be seen that after about 3 hr. the total acid reaches the level of succinic acid. Even after 24 hr. there is very little change in the succinic acid level, with the total acidity a little below it, due no doubt to some neutralization by base.

Succinic acid and hydrogen-ion production by baker's yeast after a prolonged period of oxygenation. The baker's yeast after preliminary washing was suspended in tap water, 1 kg. of yeast to 0·6 l. of tap water, and oxygenated for varying periods up to 5 days. This yeast on subsequent fermentation, under the usual conditions and in the presence of potassium chloride, gave consistently the lowest pH values reached for the suspension, this being about 1·6, though 1·4 has been observed on a few occasions. At the same time a very marked fall in the succinic acid formation occurred, with or without potassium chloride.

Fig. 5 shows the comparison of the electrometric titration curves of the outer media under the fermenting conditions described, and after an oxygenation period of 5 days. It will be seen that

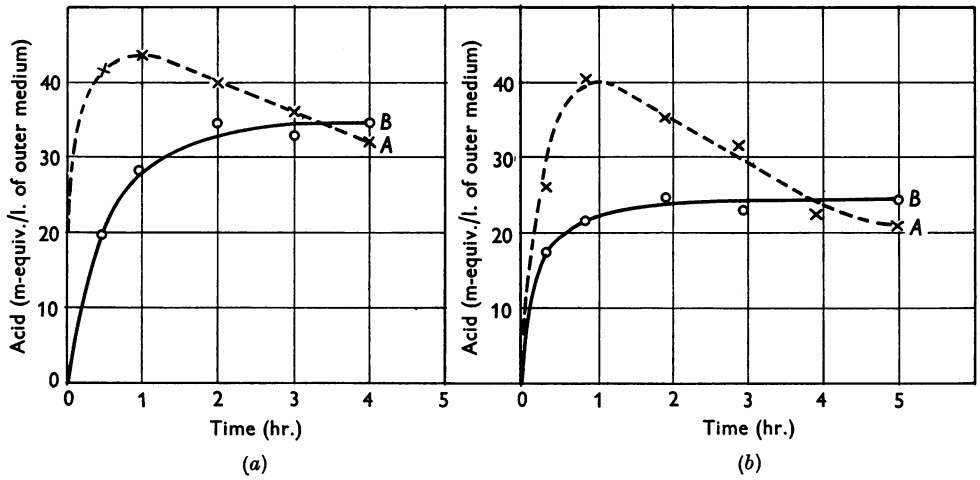

Fig. 4*a*, *b*. Mean curves of total acid (dotted lines) and succinic acid up to 4–5 hr. Conditions similar to Fig. 2. KCl included from the beginning; Fig. 4*a* shows the results with 10% (w/v) glucose; Fig. 4*b* with 5% (w/v) glucose.

Fig. 3 shows the effect of adding the potassium chloride (in the same proportion as for Fig. 2) after the fermentation has proceeded for 40 min. There is

after the 5 days' oxygenation, and by comparing with the results of Fig. 1, a very marked fall of succinic acid production has occurred and an increase

in the free acidity when potassium chloride is present in the outer medium. Retained succinic acid can here account for only a small fraction of the K^+ ions absorbed, which in turn exchange for H^+ ions.

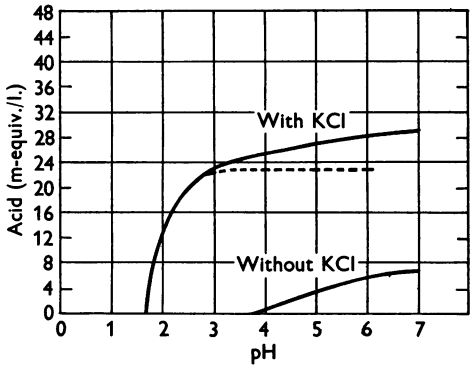

Fig. 5. Titration curves as in Fig. 1, but with yeast oxygenated for many hours prior to the fermentation.

Another experiment with oxygenated yeast, where the pH of the outer medium reached as low as 1·53, shows clearly that the K^+ ions absorbed to account for the free acid obtained on fermenting in the presence of potassium chloride, are associated with the anions of some acid other than succinic, or any such acid which can be removed by prolonged extraction with ether. Data from this experiment are given in Table 2.

could be associated with the carbonic acid system increases from about 20 % at a pH of 2·10 (*in vacuo*) to 86 % at a pH of 1·68. The lowest pH levels using potassium chloride occur with oxygenated yeast. As the amount of free acid associated with HCO_3^- in the yeast cell increases, the association with succinic acid or its anion decreases and practically disappears in some experiments, one of which was exemplified in the previous section (Table 2).

The regression equation giving the relation of excess acid-labile carbon dioxide (Y) to the hydrogen-ion concentration (X) developed (nineteen sets of observations, with a correlation coefficient of 0·84) is

$$X = 1{\cdot}15\,Y - 8{\cdot}2.$$

Thus when the excess acid-labile carbon dioxide in the yeast cells (Y) is 20 mmol./l. suspending fluid, the hydrogen-ion concentration (X) is 14·8 m-equiv./l. of suspending fluid.

The nature of the acid-labile carbon dioxide retained using potassium chloride. The introduction of samples of the fermenting mixtures into dimethylamine and dimethylamine containing barium chloride, with analyses of the centrifuged mixture, gave a measure of the amount of barium-insoluble fraction and the free carbon dioxide plus any carbamino fraction. Such experiments were carried out with group 2 of the experiments with carbon dioxide bubbling (Table 4), and are summarized in Table 5. It will be seen that the increase of acid-labile carbon dioxide is almost altogether assignable to the

Table 2. *Succinic and total ether extractable acid content of oxygenated yeast cells and suspensions*

Time of fermentation (min.)	pH of suspension	Free HCl in suspending fluid (m-equiv./l.)	Suspending fluid: Succinic acid (m-equiv./l.)	Suspending fluid: Total ether extractable acids (m-equiv./l.)	Yeast cells: Succinic acid (m-equiv./l.)	Yeast cells: Total ether extractable acids (m-equiv./l.)
0	—	0·0	0·0	1·0	1·4	13·3
24	1·53	29·6	0·0	2·9	4·6	15·2

The carbonic acid system

From the conclusions in the previous paragraph we were led to investigate the acid-labile carbon dioxide in relation to the K^+ absorption on the acid production. The total acid-labile carbon dioxide was investigated during fermentation, as described under Methods, for experiments in which the fermentation was conducted *in vacuo*, or with carbon dioxide or oxygen bubbling.

Table 3 summarizes the results obtained, and Table 4 is derived therefrom. It will be seen that for the various conditions there is a definite increase of acid-labile carbon dioxide due to the inclusion of potassium chloride during fermentation. It is least for the *in vacuo* series, and so also is the free acidity. The proportion of the free acid developed which

barium-insoluble fraction, and is thus probably HCO_3^- or H_2CO_3. Attention may be also drawn to the fact that the increase of acid-labile carbon dioxide on fermenting over that of the resting yeast is also nearly all in the barium-insoluble fraction. The increase of residual carbon dioxide which would include free carbon dioxide as well as any carbamino derivatives is relatively very small.

Effect of potassium chloride on the fermentation rate. In connexion with these experiments, it is significant to inquire if potassium chloride affects the fermentation rate. Fig. 6 shows the rate of evolution of free carbon dioxide from yeast fermenting mixtures with and without potassium chloride, set up as described under Methods. The curves may be regarded as derivative curves $d\,[CO_2]/dt$ against t of the carbon dioxide formed up to any given time. It

Table 3. *Total acid-labile* CO_2 *in yeast, resting and fermenting with and without* KCl

(All concentrations expressed as mmol./l. Suspensions and times as for Table 1.)

Conditions	No. of experiments	Acid-labile CO_2/l. suspension: Without KCl, Non-fermenting	Without KCl, Fermenting	With KCl, Non-fermenting	With KCl, Fermenting	pH of fermenting mixtures: Without KCl	With KCl
In vacuo	5	0·0	6·6	0·0	7·3	3·45	2·10
O_2 bubbling	5	0·7	4·9	0·0	7·4	3·00	1·82
CO_2 bubbling (1st series)	3	38·2	48·0	40·4	55·0	2·96	1·80
CO_2 bubbling (2nd series)	6	41·0	46·9	37·4	51·9	3·10	1·68

Table 4. *Increase of acid labile* CO_2 *due to fermentation, with and without* KCl

(All concentrations expressed as mmol./l. Suspensions and times as for Table 1.)

Conditions	No. of experiments	Increase of acid-labile CO_2 due to fermentation: Without KCl (suspension)	With KCl (suspension)	Increase of acid-labile CO_2 due to KCl inclusion (fluid outside cells)	Free acid formed with KCl inclusion (fluid outside cells)
In vacuo	5	6·6	7·3	1·3	7·5
O_2 bubbling	5	4·2	7·4	6·1	14·2
CO_2 bubbling (1st series)	3	9·8	14·6	9·1	14·9
CO_2 bubbling (2nd series)	6	5·9	14·5	16·2	20·0

Table 5. *Increase of total acid-labile* CO_2 *for the last series of experiments of Table* 4, *with data for the* HCO_3 *plus* H_2CO_3 (*total* Ba*-insoluble fraction*) *and the residual acid-labile* CO_2 *changes*

(Average of six experiments.)

	Without KCl (mmol./l. suspension)	With KCl (mmol./l. suspension)	Increase due to KCl: (mmol./l. suspension)	(mmol./l. external fluid)
Total acid-labile CO_2 increase	5·9	14·5	8·6	16·2
Increase of H_2CO_3 plus HCO_3	5·65	13·7	8·0	15·1
Residual acid-labile increase	0·3	0·85	0·55	1·0

will be seen that some difference exists. At about 30 min. carbon dioxide is being evolved somewhat more rapidly with the potassium chloride mixture. After 40 min. the reverse occurs, the evolution of gas being slower with potassium chloride. The question arises of a difference in rate of evolution accounting in some degree for the difference in retention of acid-labile carbon dioxide due to potassium chloride; but examination of the data shows that it accounts for none. Whether the mixture is examined after 30 min. when the evolution is somewhat greater with potassium chloride, or at 50 min. when it is less, the retained carbon dioxide is similar in amount. Also it may be noted that the increased retention of acid-labile carbon dioxide, due to fermenting in the presence of potassium chloride, is 140% more than when fermenting without potassium chloride, whereas the corresponding increase in rate of evolution of carbon dioxide is only 20% after 30 min., changing to a decrease after 50 min.

Other acids involved in the production of yeast acidity

For the conditions examined (unbuffered suspending media in which the cells are suspended in about their own volume of fluid) no other acid of quantitative significance, besides succinic and carbonic, was found involved during fermentation in the acidic excretion. This, however, may not be so when the conditions are much changed, and other organic acids, besides succinic, may play a relatively important role. It was found that an increase in pyruvic acid formation and excretion went parallel with the free acidic excretion (K^+ and H^- exchange), but it was quantitatively very small, being of the order of a few mg./100 ml. Similarly, phosphopyruvic acid and acetic acid were only excreted in trace amounts.

Practically negative results were obtained for the other acids examined.

DISCUSSION

In considering the quantitative relations of succinic and carbonic acids to the free H^+ ions produced, when K^+ exchanges for H^+ in fermenting yeast, these are considered here in an overall relationship, apart from the intimate nature of the mechanism involved which is dealt with later.

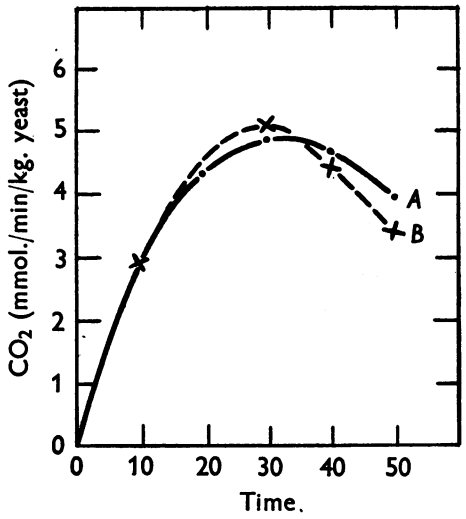

Fig. 6. Rate of emission of CO_2 from fermenting mixture, 1 part of washed centrifuged yeast to 0·6 vol. of 5% (w/v) glucose, containing 0·1M-KCl (dotted line) and without 0·1M-KCl (continuous line).

If we suppose that no organic acid is being excreted from the cell, a condition approached by prolonged oxygenation prior to fermentation, and then that potassium chloride is introduced with K^+ exchanging for H^+ ions, such H^+ ions leaving the cell might at first be considered to derive directly from the reaction

$$H_2CO_3 \rightarrow H^+ + HCO_3^-.$$

In this connexion there was evidence for the view that in decarboxylation carbonic acid was directly produced instead of carbon dioxide. The direct formation of carbonic acid seems to depend on the conditions (Conway & O'Malley, 1948; Krebs, 1948).

It was found experimentally, as shown above, that for the conditions mentioned and bubbling with carbon dioxide at atmospheric pressure, that the H^+ ions appearing outside the cells were accompanied by an increase of intracellular bicarbonate to as much as about 75% equivalence.

However, the idea of a direct origin from carbonic acid with quantitative significance is abandoned for the reason that during the fermentation the pH of the outer region of the cell, in which the K^+ and H^+ exchange was presumed to occur, would be lower than 3–4 as calculated from the distribution of the succinic system, and for other reasons. If the increased bicarbonate were then present in the outer region, the resulting concentration of carbonic acid would be far higher than could be accounted for by the carbon dioxide evolution.

As given in a following paper (see also Conway, Brady & Carton, 1949) the evidence is now very strong for the view that the increased bicarbonate arises in the inner region of the cell in a cycle of oxidation and reduction. When the catalyst is reduced in the outer region it liberates hydrogen ions, retaining electrons, and when it is oxidized in the large inner region of the cell it takes up hydrogen ions and transfers hydrogen atoms to an acceptor.

When the hydrogen ions are so removed, they are replaced by the following types of reaction

$$RCOOH \rightarrow RCOO^- + H^+ \quad (1)$$

$$RNH_3^+ \rightarrow RNH_2 + H^+ \quad (2)$$

$$H_2CO_3 \rightarrow H^+ + HCO_3^- \quad (3)$$

$$\downarrow$$

$$(\text{or} \quad H_2O + CO_2)$$

In the first two the H^+ replacement derives from the general buffering of the cells. The change of intracellular pH on fermentation under the conditions can be calculated from the data of Table 4, and from data of a previous paper (Conway & Downey, 1950), and an estimate made therefrom of the relative part played by reactions (1) plus (2) and reaction (3).

For the nine experiments with carbon dioxide bubbling, there are 47·3 mmol. acid-labile carbon dioxide/litre of mixture without potassium chloride, and 52·9 mmol./l. with potassium chloride. From the data under Table 3, it may be calculated that there are 28·6 and 44·2 mmol. HCO_3^-/l. of cell water with and without potassium chloride, giving pH values of 6·04 and 6·23 respectively. If the difference of $44{\cdot}2 - 28{\cdot}6 = 15{\cdot}6$ mmol. were quantitatively related to a corresponding free hydrogen-ion excretion, this would be expressed per litre of external fluid as $15{\cdot}6 \times 0{\cdot}36/0{\cdot}53$ (0·36 being the cell water in 1 l. of mixture and 0·53 the external water) = 10·6 m-equiv./l. of external fluid.

At the same time there is a release of hydrogen ions from the fixed buffering from a pH of 6·04 to 6·23, i.e. a change of 0·19. This fixed buffering has a value under the conditions of approximately 50 m-equiv./l. cell water/pH unit, so that 9·5 m-equiv. of hydrogen ions/l. cell water are released, or $9{\cdot}5 \times 0{\cdot}36/0{\cdot}53 = 6{\cdot}5$ m-equiv./l. external fluid. The total then of HCO_3^- and of fixed buffering contribution of hydrogen ions is approximately 17 m-equiv. This, if the view of a cyclically acting catalyst is correct, should correspond to the external free hydrogen ions, and from the pH measurements this external value is 16·5 m-equiv.

Such hydrogen ions are not of course excreted out of the cell, but express the amounts removed inside on the regeneration of the catalyst, which can then convert in turn metabolic hydrogen from the dehydrogenase into hydrogen ions.

Neither do the hydrogen ions inside the cell which are contributed to the cycle arise from the reaction, $H_2O \rightarrow H^+ + OH^-$, but from the three reactions above. It is possible, but apparently not essential, to consider reaction (2) as involving a prior addition of OH^-. In applying such changes to the gastric mucosa on acidic secretion, the HCO_3^- ions would be exchanged for Cl^-, a steady or near steady state being set up in which practically all the hydrogen ions used in the oxidative phase of the catalyst derive from carbonic acid, as in the third reaction above. This has already been pointed out for the overall change in an organic acid cyclically restored and contributing hydrogen ions to the gastric juice (Conway & Brady, 1947).

The above calculations refer to yeast which had received prior oxygenation to somewhat varying degrees, but sufficient to depress considerably the succinic acid in the cell and its excretion on fermentation. If, on the other hand, the case be taken where the yeast has not been oxygenated and ferments glucose in an unbuffered solution, then in the presence of potassium chloride the total titratable acidity is but little increased, from 44 to 46 m-equiv./l. (Fig. 1), but the succinic acid has fallen from 44 to 24 m-equiv./l. and the free hydrogen-ion concentration has risen from about 1 to 20 m-equiv./l.

Thus it would appear that the *retention* of succinic acid is nearly equivalent to the absorption of K^+ ions with corresponding rise in external hydrogen-ion concentration. Retention of succinic acid is shown by direct analyses.

If the result were such that the total titratable acidity was quite unaltered by potassium chloride, then an exact balance between retained succinic acid and free H^+ ions outside would be expected, and no *increase* occur of HCO_3^- ions specially associated with the absorption of K^+ ions, or resulting from the addition of potassium chloride.

All intermediate degrees between the extreme of no increase of HCO_3^- content with the appearance of *free* H^+ ions and an increase of up to 75% equivalence may be possible depending on the previous history of the yeast cells.

SUMMARY

1. When baker's yeast ferments 5% (w/v) unbuffered glucose, there being about an equal volume of cells to sugar solution, organic acid is excreted to about 40–50 m-equiv./l. of external fluid. Such acid is almost altogether succinic.

2. With 0·1–0·2 M-potassium chloride incorporated in the glucose solution there is but a small increase in total acid excreted, but the succinic acid is much decreased and free H^+ ions appear in roughly similar amount, usually near 20 m-equiv./l.

3. With prolonged oxygenation, 24–48 hr., before fermentation, the succinic acid excretion is greatly lessened and may almost disappear. Then very little total ether-extractable acids are found in the yeast suspension. At the same time, the maximum formation of free acid appears when potassium chloride is incorporated. A pH as low as 1·4 has been twice noted, and 1·6 frequently observed. Bubbling with carbon dioxide at atmospheric pressure, it is found that with potassium chloride, the increase in free acid is accompanied by an increase of acid-labile carbon dioxide up to about an 80% equivalence. This acid-labile carbon dioxide was shown to be HCO_3^- by the use of dimethylamine and barium chloride. As the succinic acid excreted in the presence of potassium chloride is decreased, as by prior oxygenation, the acid-labile carbon dioxide is increased in the cells relative to the control without potassium chloride.

4. For the conditions studied succinic and carbonic acids were the only acids found of quantitative significance for association, as anions, with the K^+ ions absorbed, apart from possible changes in the fixed buffering of the cells.

5. The succinic acid excreted outside the cells during fermentation remains for many hours unchanged in concentration, but the free H^+ ions formed by the exchange for K^+ practically disappear in 3–4 hr.

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Biological Production of Acid and Alkali

2. A REDOX THEORY FOR THE PROCESS IN YEAST WITH APPLICATION TO THE PRODUCTION OF GASTRIC ACIDITY

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In a previous communication (Conway & Brady, 1950) it was shown that the H^+ ions produced in the K^+ and H^+ exchange were associated with an increase of HCO_3^- ions or alternatively a retention of succinic acid. Extreme conditions could be approached when much the greater fraction was quantitatively associated with HCO_3^- or, on the other hand, with an equivalent retention of succinate. All intermediate positions are probably possible.

In the first case, with no organic acid excretion the only fixed acidity formed outside the cell is due to the exchange of K^+ and H^+ ions. In the second, the total acidity excreted is unchanged by the addition of K^+ ions.

With the fixed acidity outside the cells arising only from the K^+ and H^+ exchange, besides no appreciable excretion of organic acid, there is also no significant increase of such metabolic ether-extractable acids present within the cell. A considerable fraction of the K^+ ions absorbed appears then to be associated with HCO_3^- ions. Yet it may be concluded that carbonic acid in the outer region of the cell cannot be a quantitatively important source of the H^+ ions exchanging for K^+, since the free carbonic acid concentration is necessarily very low when the carbon dioxide output and pH of the region are considered.

The facts established in the present paper throw a fuller light on the mechanism involved, and lead to a redox theory of the origin of the H^+ ions excreted either in exchange for K^+ ions or as free organic acid.

METHODS

Measurement of external acidity. For present purposes it was unnecessary, apart from pH measurements, to make exact determinations of free organic acid. The total fixed acidity was the significant quantity. This was measured by centrifuging the sample of yeast suspension, and pipetting 0·5 ml. of the supernatant into a 25 ml. Pyrex beaker, and allowing it to stand exposed to the atmosphere for about 1 hr., if the pH were no higher than about 5, to allow any free CO_2 to escape. The escape was also usually assisted by rocking, and in some cases by heating. The sample was then titrated against phenolphthalein.

Measurement of the overall pH *of the yeast cells (evacuated of carbon dioxide).* After centrifuging, the yeast was rapidly collected into a linen bag and pressed in a hand press, shown to be efficient in expelling the interspace water to about 0–5% of the whole. The procedure was then the same as the freezing method described in a previous paper (Conway & Downey, 1950), but prior to examination the mixture was subjected to a vacuum for a short time.

For comparative purposes another method was used in which 5 g. of the pressed yeast was introduced, a little at a time into 15 ml. boiling water, the mixture cooled and made up with water to 25 ml.

Measurement of the H^+ *ions removed from the yeast cells on fermentation.* This could be obtained by titrating back the frozen, thawed and evacuated yeast to the pH of the control yeast which had not fermented glucose, but a better procedure was considered to be the following. The fixed acid in a sample of the fluid outside the cells was determined as above, then the suspension was frozen as a whole, thawed, evacuated and the pH taken. From the buffering curve obtained by electrometric titration, the amount of alkali or acid necessary to bring the pH to that of the resting cells was then determined. Usually it was very small, as the external acidity was balanced by a practically equal alkalinity produced within the cells. A similar procedure was adopted in the boiling method.

Effect of inhibitors. The effect on the acid and ethanol production of a number of inhibitors was examined in different concentrations and at different pH values. The pH was adjusted by succinate, citrate or phosphate buffers.

Yeast suspensions. For the investigation of the equivalence of alkali and acid production, the washed centrifuged yeast was suspended in the proportions of 1 kg. of washed